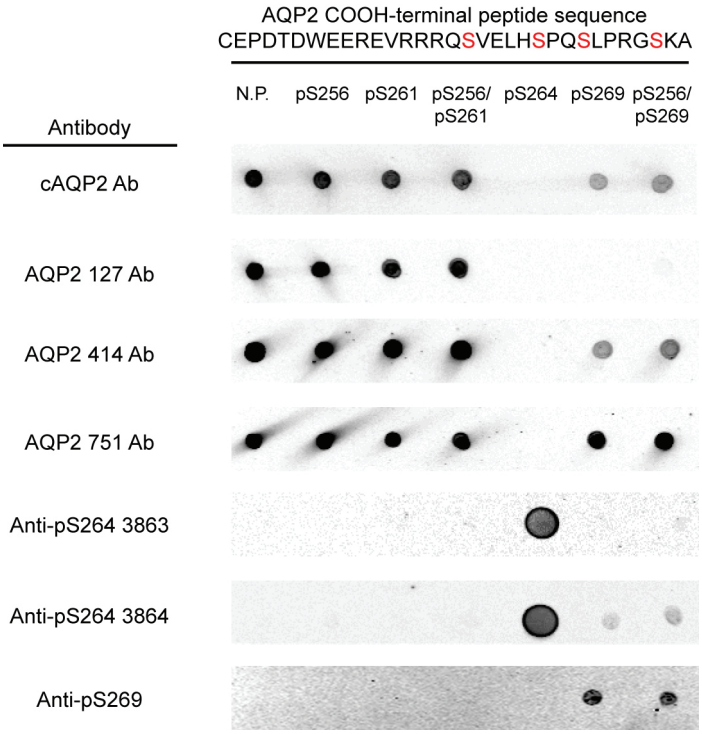
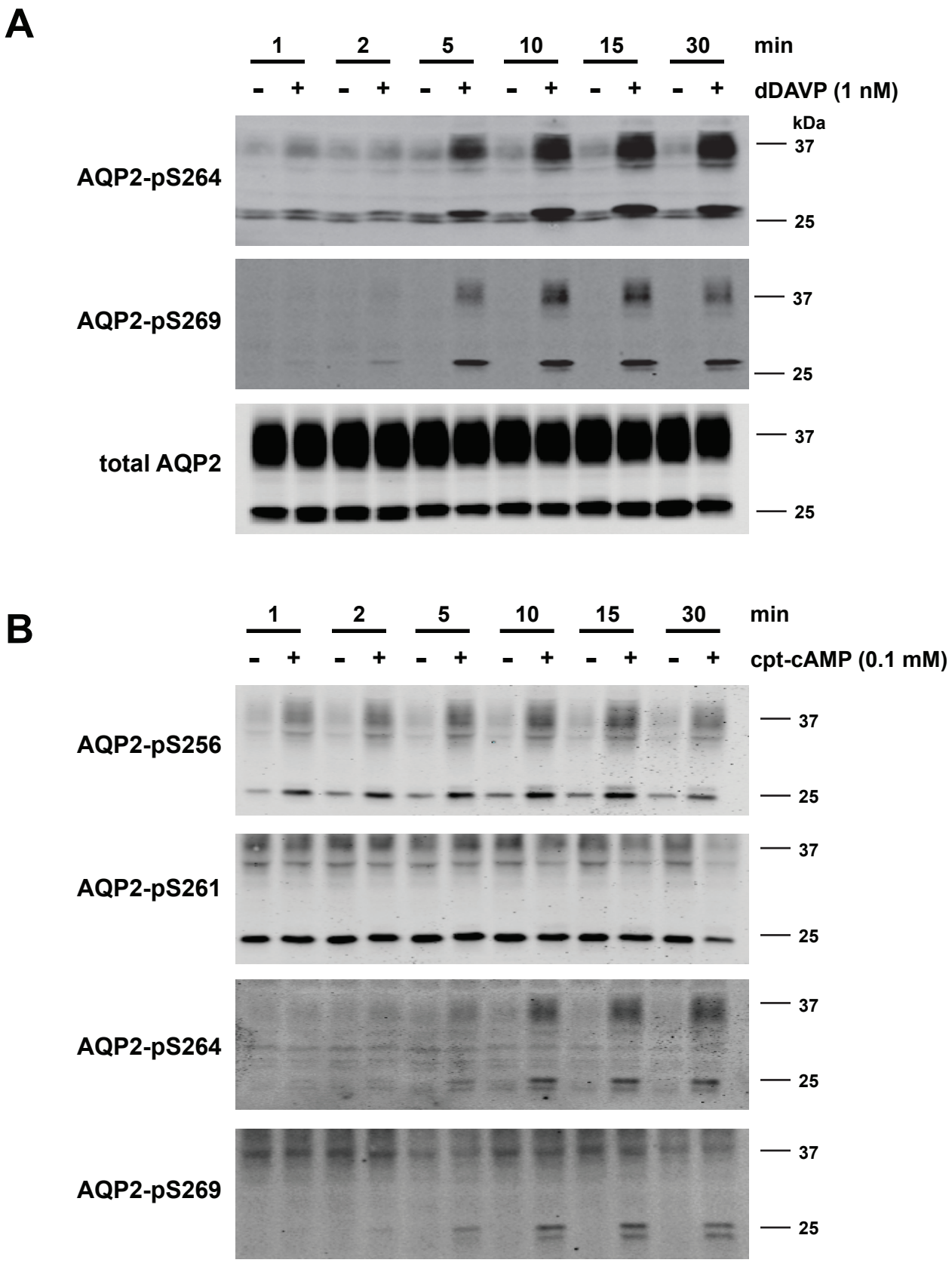
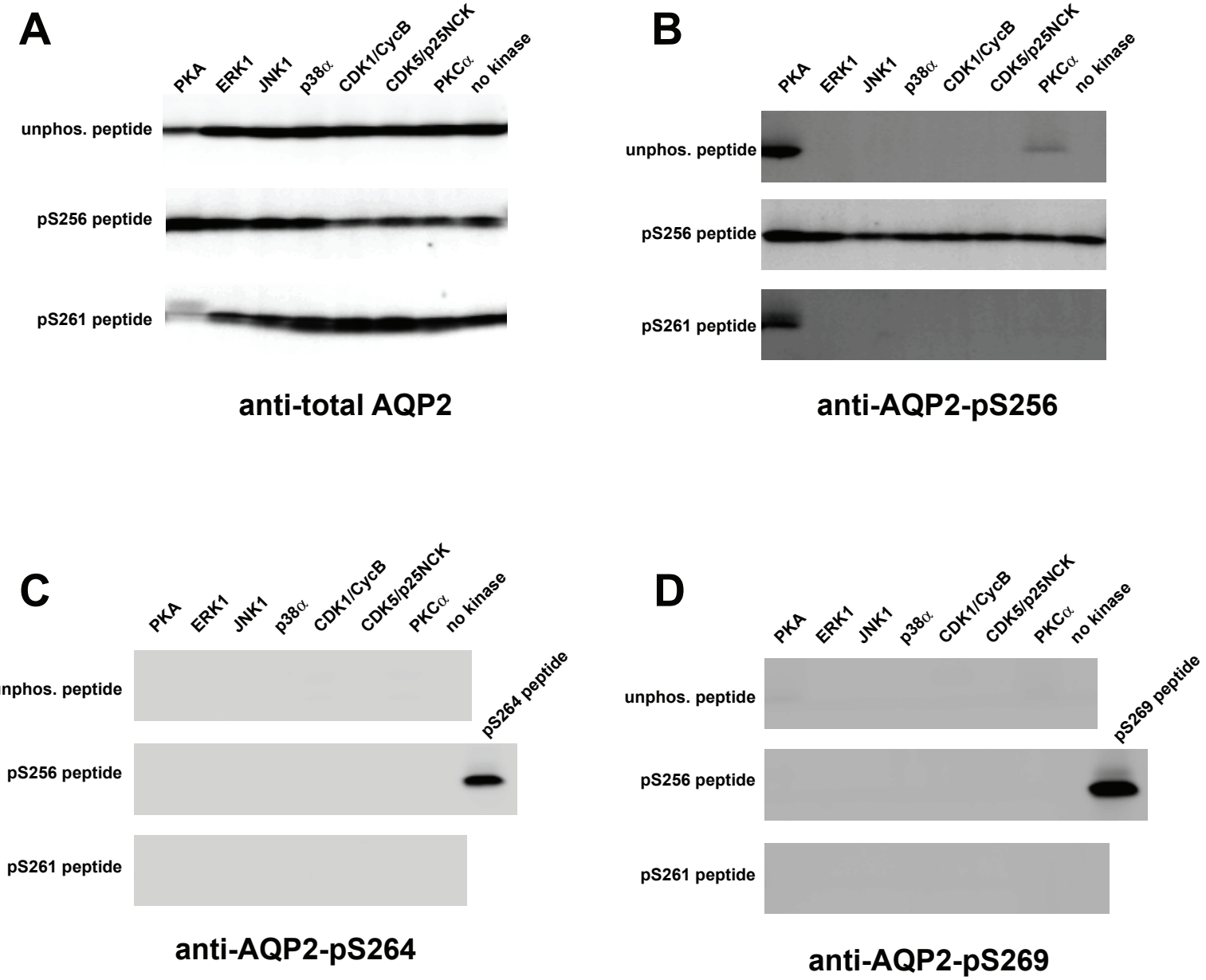


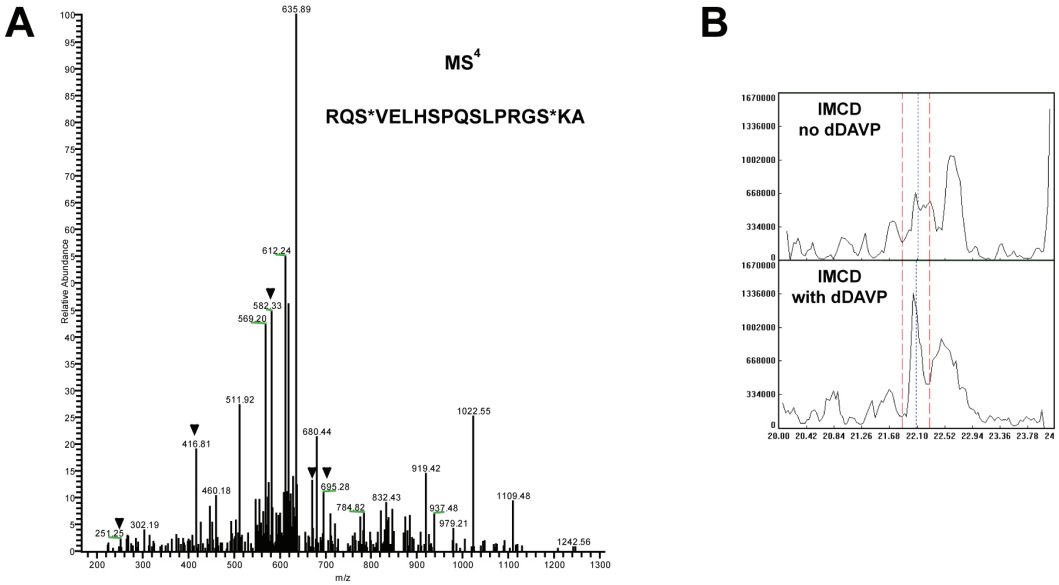
Supporting Figure 1: *Dotblots to confirm specificity of newly generated anti-pS264 and anti-pS269 AQP2 antibodies.* One microgram of various peptides were dotted and detected with indicated AQP2 antibodies. N.P., non-phosphorylated AQP2 C-terminal peptide. Antibodies to "total AQP2" (127, 414, 751, and cAQP2) are also included. Anti-pS264 and anti-pS269 antibodies are specific for pS264 and pS269 peptides, respectively.



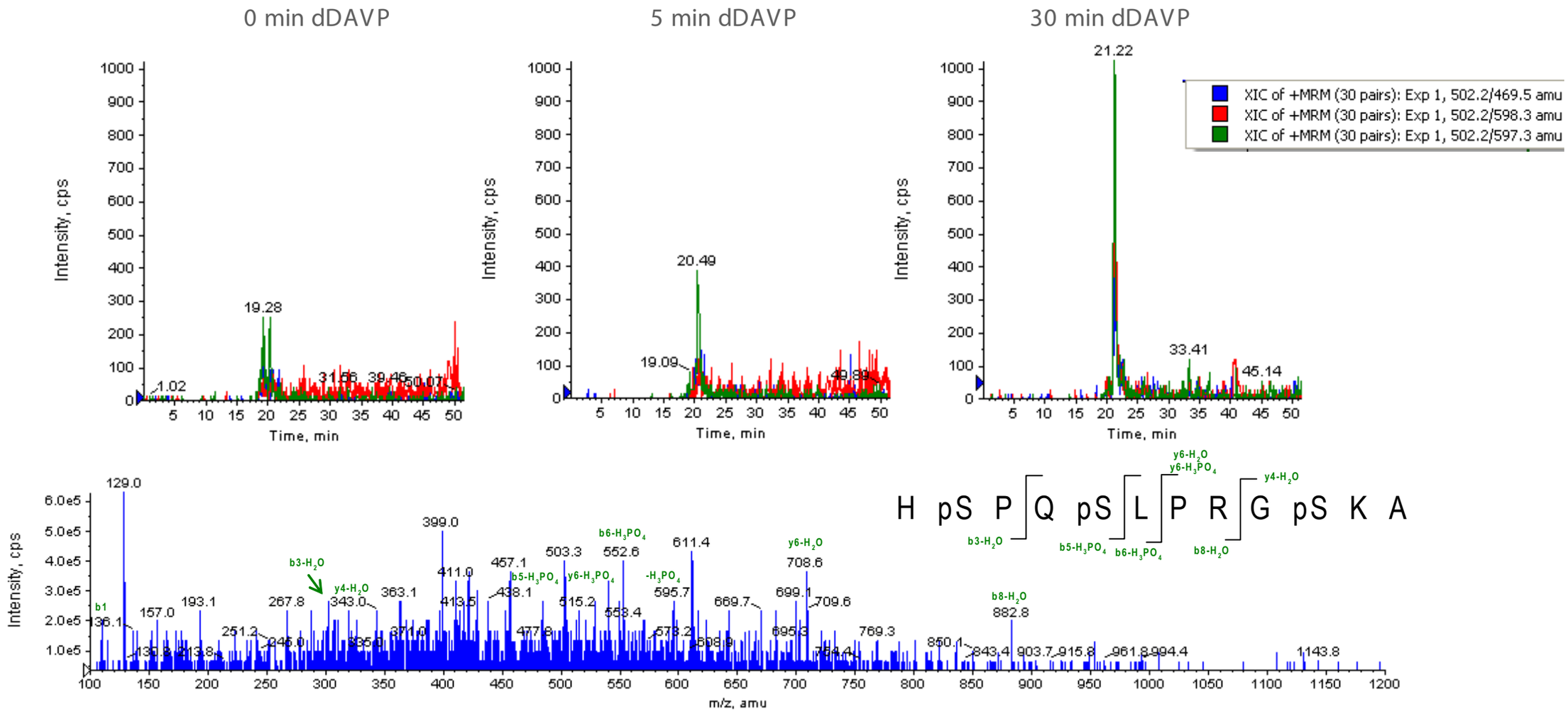
A. Time course of changes in AQP2 phosphorylation in response to 1 nM dDAVP in rat IMCD tubule suspensions. Included are representative immunoblots probed with phospho-specific antibodies recognizing pS264- and pS269-AQP2 as well as total AQP2. (Data for pS256- and pS261-AQP2 were previously reported by Hoffert et al. AJP Renal Feb;292(2):F691-700.) Control suspensions for each time point were exposed to vehicle for the same time period. B. Same timecourse as Panel A except that 0.1 mM cpt-cAMP was added instead of dDAVP. Images for all 4 phospho-AQP2 forms are included.



Kinase Assays. **Three synthetic, C-terminal AQP2 peptides (*unphos. peptide*, *p256 peptide*, and *p261 peptide*) were incubated for 1h at 30 degrees in the presence of various kinases, followed by immunoblotting and detection using the indicated anti-phospho or anti-total AQP2 antibodies. PKA phosphorylated the S256 residue of both unphosphorylated peptide as well as peptide that was already phosphorylated at S261 (*Panel B*). PKCα also phosphorylated the S256 residue very weakly but only in the unphosphorylated peptide (*Panel B*). None of the kinases tested was able to phosphorylate S264 or S269, even when S256 or S261 was already phosphorylated (*Panel C and D*). Both pS264 and pS269 AQP2 phosphopeptides were included as positive controls for antibody detection.**



Supporting Figure 4: *Identification and quantification of pS256/pS269-AQP2 by LC-MS^n analysis.* **A)** MS^4 spectrum of an AQP2 peptide phosphorylated at S256 and S269. Arrowheads indicate peaks that distinguish sites of phosphorylation. **B)** Quantification of this peptide at the MS^1 level using QUOIL software. The boundaries for peak quantification are indicated (red dashed lines).



Supporting Figure 5. MRM signals and MS/MS spectrum for a triply phosphorylated chymotryptic COOH-terminal peptide of AQP2 and response to vasopressin (dDAVP). Upper part of figure shows reconstructed ion chromatograms indicating signal for 3 different MRM transitions (indicated by colors) corresponding to the AQP2 phosphopeptide containing pS261, pS264, and pS269. Peak corresponding to this peptide is markedly increased by dDAVP at 30 minutes of dDAVP exposure. Lower part of figure shows MS^2 spectrum and corresponding sequence.